Disparate Antioxidant Enzyme Activities in Cultured Human Cutaneous Fibroblasts, Keratinocytes, and Melanocytes

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Antioxidant enzyme activities of cultured human foreskin fibroblasts, keratinocytes, and melanocytes from healthy black and Caucasian donors were measured and compared. Fibroblasts had more ($p < 0.05$) peroxidase, catalase, glutathione peroxidase, and superoxide dismutase activity than keratinocytes. Keratinocytes had more ($p < 0.05$) peroxidase, catalase, glutathione peroxidase, and superoxide dismutase activity than melanocytes. No differences in antioxidant enzyme activities were observed between the cells of any type taken from black or Caucasian people. Antioxidant enzyme activities may affect resistance to damage by oxidants induced by ultraviolet radiation and inflammation. *J Invest Dermatol 97:405–409, 1991*

Reactive oxygen species (ROS) are essential intermediates in oxidative metabolism. Nonetheless, when generated in excess, ROS can damage cells by peroxidizing lipids and disrupting structural proteins, enzymes, and nucleic acids [1]. Excess ROS are generated during a variety of cell stresses, including ischemia/reperfusion [2], exposure to ionizing and ultraviolet radiation (UVR) [3,4], and/or inflammation [1]. For protection against oxidant injury, cells possess a variety of enzymes that metabolize oxygen-reduction products [5] (antioxidant enzymes), which include, superoxide dismutase, catalase, and glutathione peroxidase.

Recently, Norris et al [6] reported that human epidermal melanocytes were more easily damaged by hydrogen peroxide (H_2O_2) than keratinocytes and that keratinocytes were more susceptible to H_2O_2 cytotoxicity than fibroblasts. The basis for these differences in susceptibility to H_2O_2 were not determined but one possibility could be that epidermal cell resistance to oxidant damage is dictated by antioxidant enzyme activities. To address this possibility, we measured peroxidase, catalase, glutathione peroxidase (GPx), and superoxide dismutase (SOD) enzyme activities in cultured fibroblasts, keratinocytes, and melanocytes obtained from Black and Caucasian donors.

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Abbreviations:

EDTA: ethylenediaminetetraacetic acid
FCS: fetal calf serum
FGM: fibroblast growth medium
GPx: glutathione peroxidase
GR: glutathione reductase
KGM: keratinocyte growth medium
KPhos: potassium phosphate buffer
MGM: melanocyte growth medium
NADPH: nicotinamide dinucleotide phosphate (reduced form)
OD: optical density
ROS: reactive oxygen species
SOD: superoxide dismutase
UVR: ultraviolet radiation

MATERIALS AND METHODS

Materials Primary keratinocyte/melanocyte cultures from neonatal foreskins were grown in keratinocyte growth medium (KGM, Clonetics Inc., San Diego, CA), which consisted of modified MCDB 153 with epidermal growth factor (10 ng/ml), insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), calcium chloride (0.15 mM), bovine pituitary extract (0.4% v/v), and antibiotics (gentamicin and amphotericin-B). Melanocyte growth medium (MGM) consisted of Ham's F-10 (Gibco, Inc., Grand Island, NY) supplemented with $NaHCO_2$ (1.2 g/l), HEPES (5.96 g/l), cholera toxin (2.5 nM), 12-0-tetradecanolyphorbol 13-acetate (TPA, 20 ng/ml), isobutyl methylxanthine (IBMX, 100 nM), and calcium chloride purchased from Sigma Chemical (St. Louis, MO) and antibiotics (penicillin/streptomycin/Fungizone) and 5% fetal calf serum (FCS) purchased from Irvine Scientific (Santa Ana, CA). Fibroblast growth medium (FGM) consisted of M199 medium (Sigma) supplemented with 10% FCS and antibiotics. Plastic tissue culture flasks were obtained from Becton Dickinson Labware, Lincoln Park, NJ. Trypsin, geneticin, melanin (isolated from sepia officinalis), and bovine serum albumin (type V) were purchased from Sigma and 0.05% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA) in Hanks' balanced salt solution was obtained from Irvine Scientific. Trichloroacetic acid and perchloric acid were obtained from Fisher Scientific.

Superoxide dismutase (SOD, from bovine erythrocytes), glutathione peroxidase (GPx, from bovine erythrocytes), cytochrome c (from horse heart), glutathione, nicotinamide dinucleotide phosphate (reduced form), xanthine, xanthine oxidase, glutathione reductase, hydrogen peroxide, and t-butyl hydroperoxide were purchased from Sigma. Bovine erythrocyte catalase was obtained from Worthington Biochemical, Freehold, NJ.

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Culture of Cells Neonatal foreskin melanocytes and keratinocytes were grown as previously described [7,8]. Calcium concentration of the KGM was 0.15 mM, which maintained the keratinocytes in a cuboidal/basaloid state of differentiation. Keratinocyte cultures that became highly squamous in differentiation were discarded. Fibroblasts were isolated and cultured from foreskin dermis as previously described [6]. Cells were incubated in a 37°C humidified environment and the culture medium was changed twice weekly. At confluence, keratinocyte/melanocyte primary cultures were separated by differential trypsinization, with keratinocytes passaged to new plates with KGM and melanocytes passaged to new plates with MGM. Fibroblast contamination of melanocyte cultures was eliminated by adding geneticin 100 μg/ml to each flask for the first 2 d after passage. All cells were further passaged 1 : 2 when 80% confluent.

Measurement of Cell Antioxidant Enzyme Activity Melanocytes, keratinocytes, and fibroblasts were used for experiments after second or third passage. Cells were harvested on the day of each experiment by scraping with a rubber policeman. Cells were then washed with 50 mM potassium phosphate buffer, pH 7.4 (KPhos), and resuspended in 0.5 ml KPhos with 1% triton-X. Subsequently, cells were sonicated on ice with a Kontes ultrasonic cell disrupter (setting 4, three five-second bursts), and centrifuged at 4°C, 10,000 × *g* for 10 min to remove cellular debris. Supernatants were then used for enzyme assays. Prior to centrifugation, 200 μl of the sonicated cell suspension was removed and assayed for DNA content by the Burton method [9] and 50 μl was removed for determination of protein content by the Lowry method [10]. All enzyme activities were expressed as unit enzyme activity per microgram DNA and per milligram protein.

Peroxidase Peroxidase activity was assessed spectrophotometrically by measuring hydrogen peroxide (H_2O_2) degradation at 240 nm, pH 7.0, at 25°C [11]. Known concentrations of bovine catalase, 100, 30, 10, 3, and 1 unit/ml were first measured to develop a standard curve. One unit of catalase activity was defined as the amount of enzyme that degrades 1 μM H_2O_2 per minute [11]. Cell supernatants were then assayed for peroxidase activity. The rate of H_2O_2 degradation of the cell samples was compared to the known catalase solutions and the amount of peroxidase activity per ml cell supernatant sample was calculated [11]. Although this method is considered specific for catalase activity [11], this has not been confirmed for human skin cells. We therefore performed the polarographic assay for catalase activity on the cell supernatants for comparison with the spectrophotometric method.

Catalase Catalase was measured polarographically as the rate of oxygen production from H_2O_2 [12,13]. For each experiment, 10 μl of known standards (determined spectrophotometrically) of bovine catalase 100, 30, 10, 3, and 1 unit/ml, were added to 400 μl of 10 mM H_2O_2 in 50 mM KPhos buffer, pH 7.0, in an airtight chamber equipped with a magnetic stirrer and maintained at 25°C by a circulating water bath. The rate of oxygen production for each catalase standard was monitored with an oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH) connected to a chart recorder (Beckman Instruments Inc., Fullerton, CA) and from this a standard curve was generated: Y-axis rate of O_2 production and X-axis catalase concentration (units/ml). The rate of oxygen production of the cell samples was compared to the known catalase standards and catalase activity (units/ml) of the cell supernatants was determined from the catalase standard curve.

Glutathione Peroxidase GPx activity of the cell supernatants was measured by assessing oxidation of glutathione by GPx utilizing tertiary-butyl hydroperoxide as substrate. This reaction is coupled to glutathione reductase (GR) oxidation of NADPH to NADP + [14]. Oxidation of NADPH was recorded using a Beckman dual beam spectrophotometer at 340 nm and 25°C. One mole of NADPH is oxidized for each mole t-butyl hydroperoxide reduced and glutathione peroxidase activity was defined as 1 U activity being 0.5 μM NADPH oxidized per minute [14].

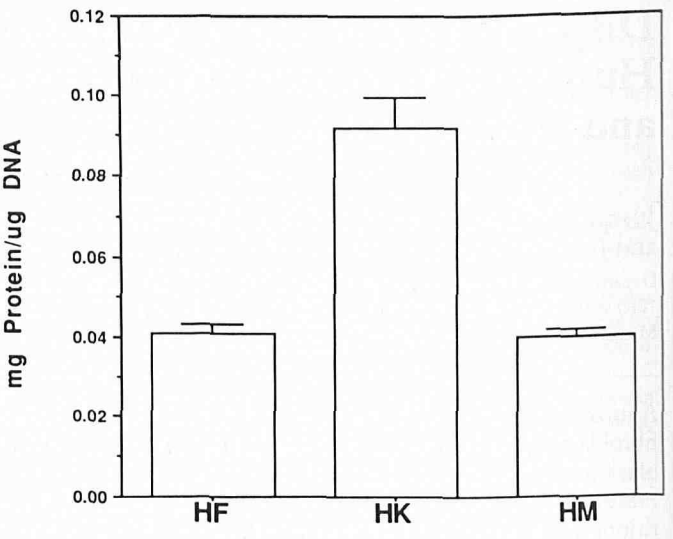

Figure 1. Ratio of mg protein to μg DNA in human fibroblasts (HF, n = 18), keratinocytes (HK, n = 15), and melanocytes (HM, n = 28). Data from Black and Caucasian people are combined. Results are reported as the mean ± SEM.

Superoxide Dismutase SOD activity was measured as the inhibition of superoxide reduction of cytochrome c via the xanthine/xanthine oxidase system [15]. With a constant source of superoxide generated by xanthine/xanthine oxidase, the rate of cytochrome c reduction by superoxide was measured on a Beckman dual beam spectrophotometer at 550 nm. One unit SOD was defined as the amount that produced 50% inhibition of the rate of cytochrome c reduction by superoxide [15]. Prior to each assay, supernatants were dialyzed (12,000 molecular weight cut-off) in 50 mM KPhos buffer to remove small molecular weight species that reduce cytochrome c and cause incorrectly low SOD activity measurements.

Total SOD activity (Mn SOD and Cu/Zn SOD) was measured at pH 7.8 and at 25°C [16]. The volume of cell supernatant necessary to inhibit the reduction of cytochrome c by 50% was determined and recorded. SOD activity per milliliter of cell supernatant was then calculated [16].

Analysis of Statistical Significance One factor analysis of variance was used to determine statistically significant differences in antioxidant enzyme activities between cell types utilizing the statistical software program SuperAnova (Abacus Concepts, Inc., Berkeley, CA). Statistical significance was accepted as $p < 0.05$. Multivariate analysis comparing the spectrophotometric peroxidase assay and the oximeter catalase assay was performed utilizing Jump statistical software (SAS Insitute, Cary, NC).

RESULTS

Each of the cell types from Black and Caucasian donors had similar mg protein/μg DNA ratios, so the data was merged. Keratinocytes had higher ($p < 0.05$) levels of cellular protein per microgram DNA compared to fibroblasts and melanocytes, which had similar amounts of protein/DNA (Fig 1). Because of the differences in protein/DNA between keratinocytes and fibroblasts and melanocytes, antioxidant enzyme activities were expressed per microgram DNA for comparisons between the cell types.

No difference ($p > 0.05$) in antioxidant activities were observed between the cell types taken from Black or Caucasian subjects (see Table I), so this data was also merged.

Each cell type had measurable peroxidase activity (Fig 2*A*). Fibroblasts had more ($p < 0.05$) peroxidase activity than keratinocytes which, in turn, had more ($p < 0.05$) peroxidase activity than melanocytes.

Epidermal cell catalase activity (Fig 2*B*), measured with the oxi-

Table I. Comparisons of Peroxidase, Catalase, Glutathione Peroxidase, and Superoxide Dismutase Activities in Human Melanocytes from Black (HMb) and Caucasian (HMc) People[a]

	Peroxidase		Catalase		Glutathione Peroxidase		Superoxide Dismutase	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
HMb	0.144	0.065–0.242 (n = 18)	0.169	0.028–0.311 (n = 10)	0.0022	0.0005–0.00484 (n = 15)	0.119	0.034–0.260 (n = 10)
HMc	0.151	0.062–0.285 (n = 20)	0.153	0.079–0.274 (n = 14)	0.0018	0.0009–0.00343 (n = 10)	0.145	0.039–0.372 (n = 10)

[a] Similar comparisons between Black and Caucasian fibroblasts and Black and Caucasian keratinocytes were performed and found to be not significant ($p > 0.05$). All results are reported as unit activity per microgram DNA. The number of individual donors tested are in parentheses.

meter, very closely mirrored the spectrophotometrically measured peroxidase activity (correlation coefficient is 0.955). Epidermal cell supernatants from the same donors were assayed in the catalase and peroxidase experiments, so direct comparison of enzyme activities was made. For each donor, all the peroxidase activity was catalase. Therefore, the spectrophotometric and oximeter assays of catalase in epidermal cells are equivalent. Complete inhibition of the peroxidase and catalase activities was attained by the addition of 10 mM sodium azide to the cell supernatants or by heat inactivation (60°C × 30 min). Melanin (50 μg/ml) had no peroxidase or catalase activity.

Although the same cell type hierarchy of enzyme activity levels persisted for GPx, GPx activity in all the cell types was lower than catalase activity (Fig 2*C*). Fibroblasts had more ($p < 0.05$) GPx

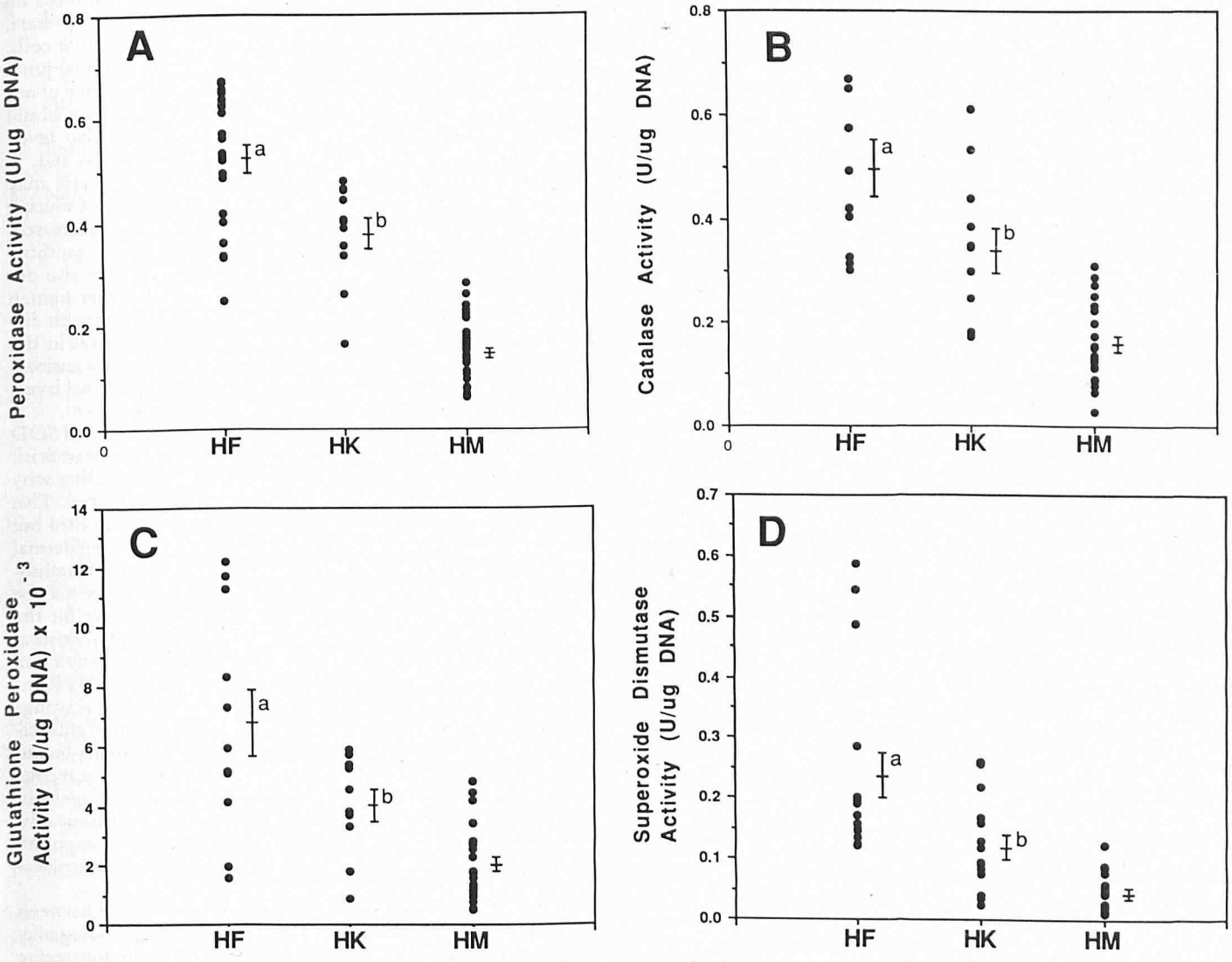

Figure 2. Antioxidant enzyme activity levels for human fibroblasts (HF), keratinocytes (HK), and melanocytes (HM). Data from Black and Caucasian people are combined. For all graphs, each *point* is the value for cells grown from a single person and each *point* represents the mean of two or three replicates. *Vertical bars,* SEM; *center horizontal bars,* mean of each data set. a, HF > HK or HM, $p < 0.05$; b, HK > HM, $p < 0.05$. *A*, peroxidase activity levels in HF, HK, and HM. Number of individual donors: HF, n = 22; HK, n = 11; and HM, n = 38. *B*, catalase activity levels in HF (n = 10), HK (n = 11), and HM (n = 24). *C*, glutathione peroxidase activity levels in HF (n = 11), HK (n = 10), and HM (n = 25). *D*, superoxide dismutase activity levels in HF (n = 17), HK (n = 15), and HM (n = 20).

activity than keratinocytes and keratinocytes had more ($p < 0.05$) GPx activity than melanocytes. All GPx activity was eliminated by heat inactivation and melanin had no GPx activity.

Fibroblasts had more ($p < 0.05$) SOD activity than keratinocytes or melanocytes (Fig 2*D*). As with the other enzymes, keratinocytes had greater SOD activity ($p < 0.05$) than melanocytes. In addition, melanocytes contained large quantities of low molecular weight cytochrome c reducing species (data not shown), which required thorough dialysis with several buffer changes to remove them. Conversely, fibroblasts and keratinocytes contained very low levels of these cytochrome c reducing species. Some of the fibroblast and keratinocyte supernatants, prior to dialysis, had no cytochrome c reducing activity. SOD activity in each cell type was completely abolished by heat inactivation and melanin had no SOD activity.

DISCUSSION

Prior experiments showed that cultured human fibroblasts are more resistant than keratinocytes or melanocytes to hydrogen peroxide cytotoxicity [6]. The present investigation extends these findings by revealing that fibroblasts have higher antioxidant enzyme activities than keratinocytes and keratinocytes have higher antioxidant activities than melanocytes. These two findings suggest that cells that have high cellular antioxidant enzyme levels may be better equipped to withstand oxidant stress.

Melanin had no detectable enzymatic antioxidant activity. All enzymatic antioxidant activities were abolished by heat inactivation. Sodium azide completely inhibited the peroxidase/catalase enzyme activity. Additionally, after centrifugation the melanocyte supernatants were clear, indicating that the supernatants were free of melanin. We therefore believe that all of the antioxidant activities were due to the enzymes themselves and not due to other nonenzymatic antioxidants.

A consistent antioxidant hierarchy was noted among the various cell types. Why this antioxidant hierarchy among the cell types exists is not clearly understood, but relatively higher peroxidase levels may be necessary to degrade the greater amounts of H_2O_2 produced by relatively higher levels of SOD. Copper/zinc SOD is irreversibly inhibited by H_2O_2 [17] and catalase [18,19] and GPx [20] are inactivated by superoxide radical. Moreover, the highly reactive and damaging hydroxyl radical is formed by the iron-catalyzed reaction of superoxide and hydrogen peroxide [21]. Therefore, to promote normal antioxidant enzyme function and ensure cell survival, "matched" activities of peroxidases and SOD working in concert, may be necessary to effectively neutralize superoxide and H_2O_2. By comparison, cells with "unmatched" antioxidant enzyme activities, for example, those with abnormally high SOD activity in association with normal or low peroxidase activities may be at risk for oxidant damage. A recent report by Seto et al [22] substantiates this possibility. Transgenic drosophila, which expressed high SOD activity, had lower survival compared to wild-type flies when exposed to the superoxide-producing pesticide, paraquat. High SOD activity of the transgenic flies readily dismutated the paraquat-generated superoxide, thus producing H_2O_2 levels that exceeded the peroxidase defenses of the flies, culminating with toxicity and death. Other studies with SOD-overproducing *Escherichia coli* [23–25] and mammalian cells [26] have shown increased sensitivity to paraquat.

The antioxidant hierarchy among the cell types may also be a manifestation of growth of the cells in different culture media. Each of the cell types was grown in defined, standard media for optimal growth of the cells. One single type of media does not, to our knowledge, exist that will support optimal growth of all three cell types. Melanocytes do manifest ample growth when co-cultured with keratinocytes in KGM but fibroblasts do not. We are actively investigating the issue of culture media effects on human skin cell antioxidant enzymes. In preliminary experiments we have found that antioxidant levels of fibroblasts, keratinocytes, and melanocytes can be altered by different culture media additives but the hierarchy of antioxidant enzyme activities among the three cell types remains the same.

Catalase was the predominant peroxidase enzyme in all the cell types. However, just comparing catalase and glutathione peroxidase activities according to their in vitro abilities to degrade H_2O_2 is incomplete. The distribution of catalase and GPx in human epidermal cells is not known, but in mammalian cells catalase resides in peroxisomes and GPx predominates in the cytoplasm and mitochondria [5]. Furthermore, the rate constant for the H_2O_2/GPx reaction is more than twice that of the rate constant for the formation of catalase compound I (5.0×10^7 $M^{-1}s^{-1}$ versus 1.7×10^7 $M^{-1}s^{-1}$, respectively). Both catalase and GPx neutralize H_2O_2, however, GPx also neutralizes lipid peroxides [5]. From these differences in enzyme location, the rate at which the enzymes react with H_2O_2 and the type of hydroperoxides neutralized by the enzymes, there has been considerable debate as to which is the primary enzyme responsible for the regulation of intracellular H_2O_2. In most cells there is co-operation between catalase and GPx function. Catalase readily neutralizes high peroxisomal H_2O_2 concentrations but some H_2O_2 does escape [5]. This H_2O_2 is quickly neutralized by cytosolic GPx [5]. Finally, a variety of cellular factors exist that regulate catalase and GPx levels [5]. The mechanisms controlling catalase and GPx levels in human skin cells have yet to be determined.

Keratinocyte antioxidant activities were consistently intermediate between fibroblasts and melanocytes. We chose to focus on keratinocytes that were in a less-differentiated, basaloid state, thereby allowing us to study the actively dividing progenitor cells. Damage to the progenitor cells along the dermal-epidermal junction would affect the entire epidermis; therefore knowledge of antioxidant activity levels in the basaloid state of differentiation would help us to understand antioxidant defense capacity at that level. Keratinocytes differentiate as they traverse the epidermis and, as they differentiate, their antioxidant enzyme activity levels may change. Reiners et al [27] reported that catalase activity of murine keratinocytes increased as stages of differentiation increased. Reiners and Rupp later reported that murine keratinocyte xanthine oxidase and xanthine dehydrogenase activity levels were also dependent upon keratinocyte differentiation [28]. Whether human keratinocytes increase their antioxidant enzyme activities with differentiation has not yet been established but keratinocytes in the outermost layers of the epidermis may require the highest antioxidant defense capability in order to shield the lower epidermal layers from environmental oxidant stress.

Human melanocytes had lower "matched" peroxidase and SOD activities than keratinocytes and fibroblasts that correlated with their extreme sensitivity to damage by H_2O_2. It is puzzling why melanocytes would possess such low antioxidant activities. This may be solely a manifestation of melanocytes grown in vitro but other explanations are intriguing. Melanocytes are the epidermal cells that produce the pigment melanin for distribution to keratinocytes. The majority of the melanocyte's metabolic energy may be committed to the synthesis of melanin with little reserve for the production of antioxidant enzymes. Alternatively, melanin exists as a stable free radical [29], can readily participate in oxidation and reduction reactions [30–32], and acts as a scavenger of UVR-induced ROS [33,34]. Melanocytes may rely on the radical scavenging ability of melanin rather than produce large amounts of antioxidant enzymes. However, melanin is stored in membrane-bound cytoplasmic vesicles and may not be available to freely scavenge non-melanosomal toxic oxygen products. Therefore, melanin would not be protective against cell membrane or cytoplasmic oxidant insults, which would be a disadvantage during acute exposure to excess oxidants such as during severe inflammation of the epidermis.

No difference in antioxidant activity levels was noted between cell types cultured from Black or Caucasian skin. This finding may not be of importance for human fibroblasts as they do not receive doses of UVR equal to keratinocytes or melanocytes [35], nor do they acquire melanin from melanocytes. However, keratinocytes and melanocytes that are exposed to higher UVR levels and contain melanin may be influenced by the products of UVR/melanin photochemical reactions. This is especially important for Caucasian

epidermis, which is an inferior UVR filter [36] and contains potentially phototoxic pheomelanin [37,38]. Exposure of Caucasian epidermis to UVR may generate excess free radicals that overwhelm the antioxidant capacity of keratinocytes and melanocytes thereby damaging cell proteins, membranes, and DNA. Although UVR's carcinogenic effects are mediated mostly by direct DNA damage, the UVR-generated ROS are an additional factor in epidermal cell carcinogenesis [39,40]. Black keratinocytes and melanocytes, which contain eumelanin, may not require high antioxidant enzyme levels to guard against UVR-induced ROS due to the superior UVR filtering and ROS neutralizing capability of eumelanin. Ultimately, Black epidermis may be less likely than Caucasian epidermis to develop skin cancer not only because of the excellent UVR-filtering ability of eumelanin but also because of eumelanin's radical-scavenging ability.

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